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CHROMOSOMA

Rapid and High Resolution Detection of in situ Hybridisation to Polytene Chromosomes Using Fluorochrome-labeled RNA*

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Abstract. Fluorochrome-labeled RNA allows the rapid detection of in situ hybrids without the need for long exposure times as in the autoradiographical hybridisation methods. Resolution is high because of the high resolving power of fluorescence microscopy. The application of a previously reported method for the hybrido-cytochemical detection of DNA sequences to polytene chromosomes of *Drosophilia* is described. – The specificity and sensitivity of the method are demonstrated by the hybridisation with polytene chromosomes of 1) rhodamine-labeled 5S RNA, to the 5S rRNA sites of *D. melanogaster* (56F) and *D. hydei* (23B), 2) rhodamine-labeled RNA complementary to a plasmid containing histone genes, to the 39DE region of *D. melanogaster*, 3) rhodamine-labeled *D. melanogaster* tRNA species (Gly-3 and Arg-2), to their respective loci in *D. melanogaster*, 4) rhodamine-labeled RNA complementary to the insert of plasmid 232.1 containing part of a *D. melanogaster* heat shock gene from locus 87C, to *D. hydei* heat shock locus 2-32A. In the latter instance it was possible to demonstrate the labeling of a double band which escaped unambiguous detection by autoradiography in the radioactive cytochemical hybridisation procedure because of the low topological resolution of autoradiograms. – The sensitivity of the fluorochrome-labeled RNA method is compared with the radioactive methods which use ^{3}H- or ^{125}I-labeled RNAs. The factors governing the sensitivity and the number of bound fluorochrome molecules to be expected are discussed.

Introduction

The use of in situ hybridisation to detect specific genes in polytene chromosomes of *Drosophila* has found wide application. For several defined RNA species the unequivocal localisation of the coding DNA in one or several distinct bands of the chromosomes was found. Well known examples are the localisation of 5S

* Dedicated to Professor W. Beermann in honour of his 60th birthday

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ribosomal RNA genes in *D. melanogaster* (band 56F) (Wimber and Steffenson, 1970) and in *D. hydei* (band 23B) (Alonso and Berendes, 1975), and the 9S histone mRNA genes in *D. melanogaster* (39DE) (see Pardue et al., 1977). Genes coding for rRNA have been localised in the nucleolus (Pardue et al., 1970; Alonso, 1973). Several tRNA species have been assigned to specific bands (for references see Hayashi et al., 1980).

In situ hybridisation has also been used to study transcriptional control of specific mRNAs after, e.g., heat shock (see Spradling et al., 1975; Lubsen et al., 1978 and references cited there) or ecdyson stimulation (Bonner and Pardue, 1976). The in situ hybridisation patterns of these RNAs were found to correlate with the puffing pattern induced by the same treatments.

The in situ hybridisation technique using radioactively labeled nucleic acids and autoradiography to detect the hybrids has its shortcomings. In order to keep the exposure time of autoradiograms within reasonable limits nucleic acids of high specific activity have to be be used. Especially with RNA obtained in vivo this is often difficult to achieve. In vitro ^{125}I labeling of RNA can be used to obtain high specific activities but has the disadvantage of lower autoradiographical resolving power due to the high energy of the desintegration (Prensky et al., 1973).

These problems can be overcome by the use of fluorochrome-labeled RNA which makes the in situ hybrids promptly visible in the fluorescence microscope and produces images with high spatial resolution (Bauman et al., 1980, 1981a, b)

The advantages of fluorescence microscopical detection of in situ hybrids have also been described by Rudkin and Stollar (1977). They used an antibody raised in rabbits which was specific for RNA·DNA hybrids to detect 5S rRNA hybridised to the 56F locus of *D. melanogaster*. The bound antibody was visualised with a second fluorochrome-labeled antibody specific for rabbit immunoglobulins. The general adoption of this elegant method to detect in situ hybrids seems to be retarded by the difficulty to obtain a specific anti-hybrid antiserum.

The aim of the investigations described in this paper was to demonstrate the usefulness of fluorochrome-labeled RNA for the immediate fluorescence microscopical detection of in situ hybridisation to polytene chromosomes of *D. melanogaster* and *D. hydei*. We used 5S RNA from *D. hydei* tissue culture cells, two species of purified *D. melanogaster* tRNA, RNA complementary to the DNA of a clone containing histone genes and RNA complementary to clones containing *D. melanogaster* heat shock genes. With these RNAs we intended to give further demonstration of the specificity, the sensitivity and the resolving power of the in situ hybridisation method employing fluorochrome-labeled RNAs.

Materials and Methods

Escherichia coli tRNA, RNase A and RNase T1 were obtained from Boehringer, Mannheim. Sephadex-G50 from Pharmacia Fine Chemicals, Uppsala, Sweden and TRITC (crystalline, isomer R) from Baltimore Biological Laboratories, Div. Becton, Dickinson and Co., Cockeysville, Maryland. ^{3}H-ATP (25–40 Ci/mmole) and ^{3}H-UTP (35–50 Ci/mmole) were obtained from New England Nuclear. All other chemicals were of analytical grade.

1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; actetate buffer contains 50 mM sodium acetate, pH 5.0, 5 mM EDTA; 70% formamide/3 × SSC is formamide-10 × SSC, 7/3 (v/v); PBS is 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2.

Synthesis of Tetramethylrhodamine Thiosemicarbazide. 1 mg TRITC, dissolved in 1 ml dimethylsulfoxide (DMSO) + 10 µl pyridine, was mixed with 20 µl of a 0.6% solution of hydrazine in DMSO. After a reaction time of 4 h at room temperature in the dark, 10 µl acetic acid were added. This reaction mixture was used without further purification and it was found to be stable at −30° C giving the same labeling results over a period of several months (Bauman et al., 1981a).

Synthesis of Complementary RNAs. cRNAs were synthesised in vitro with *E. coli* DNA-dependent RNA-polymerase essentially as described by Steinert et al. (1973). Synthesis and yield of cRNA was monitored by the amount of acid precipitable ^3H label obtained. The size of the cRNAs, estimated from their chromatography behaviour on Sephadex columns and the obtained degree of fluorochrome labeling, was 40–200 nucleotides.

RNAs Used. 5S RNA was prepared from *D. hydei* tissue culture cells (Sondermeijer and Lubsen, 1979) by neutral sucrose/dodecylsulfate gradient-centrifugation. The 3–7S fraction was used. It contained approximately 2–3% 5S rRNA.

Histone cRNA was cRNA prepared using clone cDm500 containing the histone genes of *D. melanogaster* (Lipton et al., 1977) as template. About 57% of the plasmid DNA is histone DNA. The lenght of the insert is 8.6 kbp (kilo base-pairs) and it containes 1.8 times the complete histone gene repeat unit. Complementary RNA of this clone hybridised to regions 39DE of *D. melanogaster* (Lipton et al., 1977).

232.1 cRNA was prepared using the DNA from clone 232.1 which containes 1.0 kbp (20% of the plasmid) of *D. melanogaster* chromosomal DNA from locus 87C one of the heat shock loci (Livak et al., 1978). cRNA from this clone hybridises to locus 2-32A and (to a lesser extent) 2–36A of *D. hydei* (Peters et al., 1980).

232.1 insert cRNA was prepared from the same clone, but here only the insert DNA was used as template.

244.1 cRNA is prepared using the pPW244.1 subclone DNA as template. This clone contains 0.8 kbp (15% of the plasmid) *D. melanogaster* chromosomal DNA from locus 63BC (Holmgren et al., 1979) one of the heat shock loci. Its cRNA hybridises with locus 4–81B of *D. hydei* (Peters et al., 1980).

tRNA(Gly-3), tRNA(Arg-2) and total tRNA of *D. melanogaster* were isolated as described by Dudler et al. (1980). The amino acid charging values obtained were 1400 pM/A260 for both pure tRNAs. The lower than optimal charging (1700 pM/A260) obtained is probably due to the unstability of the *Drosphila* enzymes.

Fluorochrome-Labeling of RNA. The fluorochrome-labeling of the RNA was performed as follows (Bauman et al., 1981a, b): 1–5 µg of RNA in 50 µl acetate buffer were oxidised with 10 µl $NaIO_4$ solution (200 mg/ml in water) for 1 h at room temperature in the dark. Five minutes after the addition of 10 µl ethylene glycol the oxidised RNA was purified by chromatography on a 1-ml column of Sephadex-G50. To the 200 µl peak-fraction 2 µl of the thiosemicabazide reaction-mixture were added. After 4 or 16 h excess dye was removed by phenol extraction and the labeled RNA was ethanol precipitated after the addition of *E. coli* tRNA as carrier.

The degree of labeling was determined when possible by absorbance measurements (carrier RNA was omitted in this case) using $E(555\,nm) = 48{,}000\,M^{-1}\,cm^{-1}$ for bound rhodamine (Van Dalen and Haaijman, 1974) or by comparing the fluorescence of dilutions of rhodamine-labeled *E. coli* tRNA with known degree of labeling. For *E. coli* tRNA (which we use as an internal control in the labeling procedure) a degree of labeling of 70–90% was routinely obtained.

In situ Hybridisation Procedure. Squash preparations of salivary gland polytene chromosomes of *D. melanogaster* and *D. hydei*, fixed in ethanol-acetic acid (3/1, v/v), were used. Conditions for the in situ hybridisation as described by Lubsen and Sondermeijer (1978) and as modified for fluorochrome-labeled RNA as described by Bauman et al. (1981a, b) were used. Essential for good results with fluorochrome-labeled RNA is that the hybridisation is performed at low temperatures in formamide-containing buffers. All hybridisation reactions were performed at room temperature (23° C) with 2–8 µl RNA solution in 70% formamide/3 × SSC. The fluorochrome-RNA bond was found to be stable under these conditions. We found a strong dependency of the stability of the fluorochrome-RNA bond on temperature and formamide concentration (Bauman et al., 1981a, and Palings and van Duijn, unpublished observations).

In general the hybridisation time used was 66–70 h. RNA concentrations ranged from 2 to 1000 µg/ml as indicated in the text.

We found it to be essential for good results that the hybridisation reaction was started immediately after the RNase and denaturation pretreatments. The use of preparations stored dry for some time after the denaturation and dehydration gave very poor results.

The complete hybridisation procedure used is the following: slides were treated with a few drops of RNase solution (100 µg RNase A + 1 µg RNase T1 per ml 2 × SSC) in a moist chamber for 2 h, washed with a large amount of 2 × SSC (3 changes in 1.5 h), dehydrated with 70%, 90% and 100% ethanol and air dried. Denaturation was performed with freshly prepared 0.07 M NaOH for 3 min followed by ethanol dehydration and air drying as above. When dry, 2–8 µl RNA solution in 70% formamide/3 × SSC were placed on a small cover-glass (15 × 15 mm) which was then inverted on the slide. No sealing was used, but the slides were incubated in a moist chamber containing paper tissues saturated with 70% formamide/3 × SSC.

After 66–70 h the cover-glasses were rinsed off with 70% formamide/3 × SSC and the slides were washed in 70% formamide/3 × SSC (2 changes in 0.5 h) and dehydrated with 70% and 90% ethanol, each containing 0.3 M ammonium acetate (Brahic and Haase, 1978), 100% ethanol and air dried. For microscopical observation and microphotography slides were mounted in 10 µl PBS containing 1 µg 4′,6-diamidino-2-phenyl indole (DAPI) per ml to stain the DNA (Lin et al., 1977). After observation the coverslips were removed with a jet of methanol and after air drying the slides were stored at 4° C in the dark. We found that in this way the slides could be stored for many months without loss of fluorescence.

Microphotography and Band Mapping. For mapping purposes photographs were taken of the blue DAPI-fluorescence and the red rhodamine fluorescence of the same field and at the same magnification. When prints of the negatives were superimposed over a strong light-source, whereby the weak uniform background fluorescence of the chromosomes helped in the accurate positioning, it was easy to identify the labeled bands. It might be of help to add to an RNA preparation some rhodamine-labeled RNA (e.g., a purified tRNA species) with known location as a positive control and aid in mapping unknown RNA sites.

Photographs were taken with a Leitz Orthomat on an Orthoplan microscope with Ploemopak epi-illuminator on 35 mm Kodak Tri-X Pan film exposed at 400 ASA. The filter combinations were SP560 + BG38 + 2 mm LP530 excitation filters, 580 nm dichroic mirror and LP590 emission filter for the red rhodamine fluorescence and 4 mm UG1 excitation filter 400 nm dichroic mirror and LP435 emission filter for blue DAPI fluorescence.

Results

5S RNA

D. hydei 5S RNA was hybridised to *D. hydei* and *D. melanogaster* polytene chromosomes at concentrations of 1–20 µg/ml. For *D. hydei* chromosomes after hybridisation to an R_0t value of 16 the band at 23 B(1) (as determined using the map of *D. hydei* polytene chromosomes drawn by Berendes (1963)) was found consistently brightly labeled (Fig. 1). This confirms the previous localisation of the 5S rRNA site at 23B(12) by Alonso and Berendes (1975) using ^{3}H-labeled 5-7S RNA and autoradiography.

Besides this bright band at 23B1 we found many extra bands labeled (Fig. 1b). The number and the intensity of these extra bands became less prominent with lower R_0t values ($R_0t = 3$, Fig. 1d). They were absent at the low R_0t values at which the 5S band is apparently still saturated. No attempts were made to exactly map the labeled bands, but a detailed comparison of a number of photographed nuclei revealed that the labeling pattern was unique and the relative fluorescence intensities of the bands were comparable in different nuclei on one slide and in nuclei from different experiments. We do not know much about the exact nature of the RNA producing the extra labeled bands. Some of the bands might be tRNA sites

Fig. 1 a–
an R_0t v
fluoresce
23 B. In

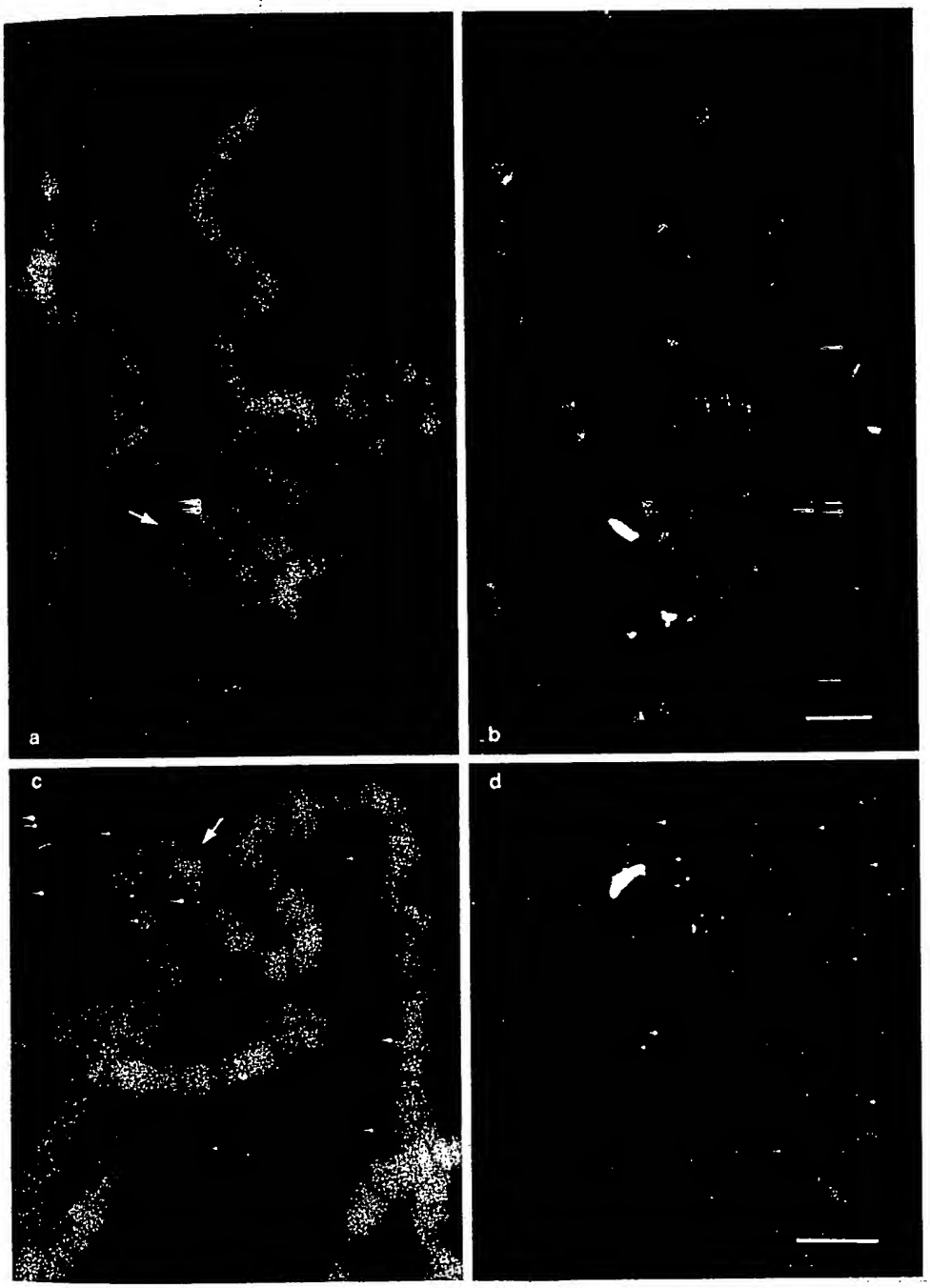

Fig. 1a–d. Hybridisation of rhodamine-labeled *D. hydei* 5S RNA to *D. hydei* polytene chromosomes to an R_0t value of 16 (**a, b**) and 3 (**c, d**). From the same fields photographs were taken of the blue DAPI fluorescence (**a, c**) and the red rhodamine fluorescence (**b, d**, respectively). *Arrow* indicates the band 23B. In Fig. 1–6 the bars represent 10 μm

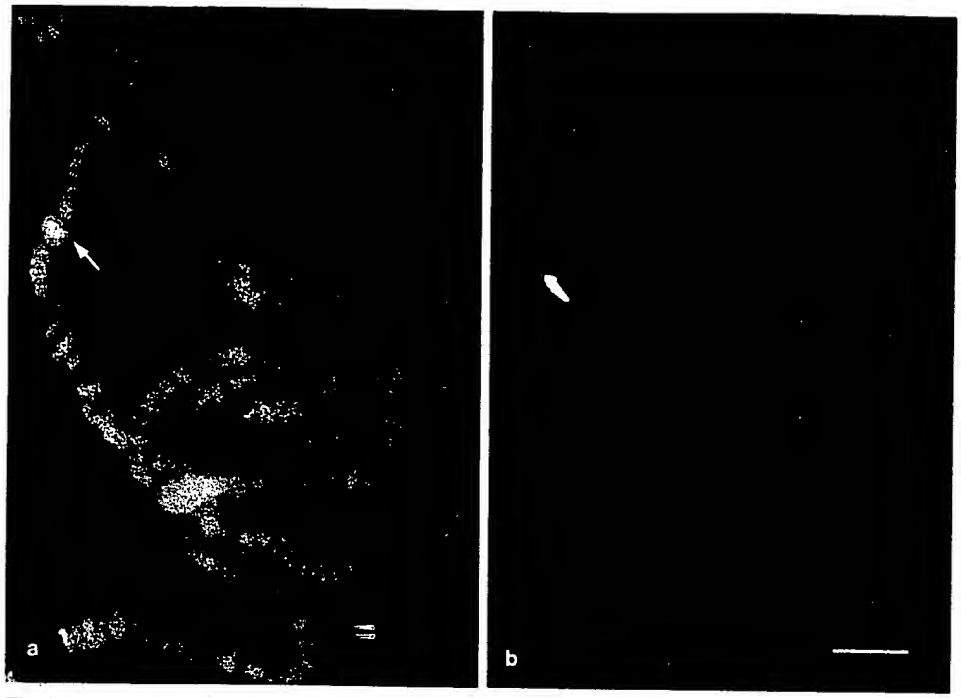

Fig. 2a and b. Hybridisation of rhodamine-labeled *D. hydei* 5S RNA to *D. melanogaster* polytene chromosomes to an R_0t value of 3. Blue DAPI fluorescence (**a**) and red rhodamine fluorescence (**b**) are from the same field

(compare the results with total tRNA, below) while many others might be caused by degradation products of specific mRNAs. At high R_0t values the nucleolar DNA was also found to be clearly labeled.

For *D. melanogaster* chromosomes we found that after hybridisation of rhodamine-labeled *D. hydei* 5S RNA the 56F band (the known site of the 5S rRNA genes (Wimber and Steffensen, 1970)) was very brightly fluorescent (Fig. 2). The RNA concentration used was 4 µg/ml and hybridisation was to a R_0t value of 3. Occasionally at this R_0t value a few other bands were found to fluoresce very faintly but the brightnes of labeling of these other bands was low as compared to the homologous *D. hydei* preparations to which 5S RNA had been hybridised to comparable R_0t values.

Hybridisation of tRNAs to D. melanogaster Polytene Chromosomes

Rhodamine-labeled total tRNA and the purified tRNA (Gly-3) and tRNA (Arg-2) of *D. melanogaster* were hybridised to *D. melanogaster* polytene chromosomes.

Total tRNA, hybridised to a R_0t value of 10 gave labeling of 3 bands of which 56F and 42A were clearly fluorescent and a third, probably 29D was faintly fluorescent (not shown). In addition to these three, 5–7 bands (not mapped) were faintly fluorescent when the hybridisation was to an R_0t of 16. In an attempt to localise more tRNA genes with this total tRNA preparation, hybridisation to R_0t of

1800 was performed, but this resulted in an extremely high overall fluorescence due to binding of detached fluorochrome molecules to the preparations (Bauman et al., 1981a). Because of this, specific fluorescence of bands could not be observed.

The observed small number and the distribution of labeled sites therefore probably reflects the local relative number of tRNA genes and/or the abundance of different tRNA species in total tRNA. Alternatively, the hybridisation rate might have been lower than assumed under our conditions (see discussion).

tRNA(Arg-2) was previously localised, using ^{125}I-labeled RNA and autoradiography, at three sites: 42A, 84F and 56EF (Hayashi et al., 1980). We found all these three loci consistently labeled after hybridisation of rhodamine-labeled tRNA(Arg-2) to a R_0t value of 26 (Fig. 3) although the intensity at 56EF was always much higher than the intensities at 42A and 84F, which were comparable to each other. The fluorescent band in the 42A region was faint and dispersed in appearance when the region was puffed (compare Hayashi et al., 1980), but sometimes it was present as a sharp band (2 out of 7 photographes examined). In addition to these three loci we also found a sharp band at 62A and in the 53A region (probably band 52F) a faint but distinct fine band in most nuclei. This correlates well with the localisation of tRNA(Glu-4) at loci 52F, 56F and 62A (Kubli and Schmidt, 1978). From this it might be concluded that tRNA(Glu-4) is the main impurity in this tRNA(Arg-2) preparation or that a high degree of homology exists between these two tRNA species (see discussion).

Finally a very faint labeling was repeatedly found in the 85AB and 90B-D region.

We did not perform competition experiments with unlabeled 5S rRNA to eliminate the possibility that some 5S rRNA was present as an impurity in the tRNA(Arg-2) preparation.

tRNA(Gly-3), rhodamine-labeled, gave after hybridisation to a R_0t value of 20 up to twenty fluorescent bands (Fig. 4). With tRNA(Gly-3) the most conspiciously labeled site was 56F. Band 57C, often present as a dot-like band (Fig. 4b) was also always labeled. The other Gly-3 sites (22BC, 28D, 35BC, 53E and 55E, Hayashi et al., 1980) were on close examination often found to be labeled. This labeling however was much weaker than several other sites to which Gly-3 or any other tRNA was not assigned previously, notebly 99A, 96A and 92AB (Fig. 4b). Also the nucleolar DNA was found to be slightly labeled. This might indicate that this tRNA preparation contained more impurities than tRNA(Arg-2), were up to nine bands were found labeled, or that a higher homology in base sequence of this tRNA with other tRNA species exists (see discussion).

Hybridisation of rhodamine-labeled Arg-2 or Gly-3 tRNAs to R_0t values of 450–600 resulted in an increased background fluorescence caused by the binding of detached fluorochrome molecules to the proteins in the preparation. In spite of the high background fluorescence, the specific fluorescence of the labeled sites still remained visible (not shown).

Histone cRNA

Rhodamine-labeled histone cRNA, hybridised to an R_0t value of 3.4 to *D. melanogaster* polytene chromosomes, gave an extremely intense fluorescent 39DE

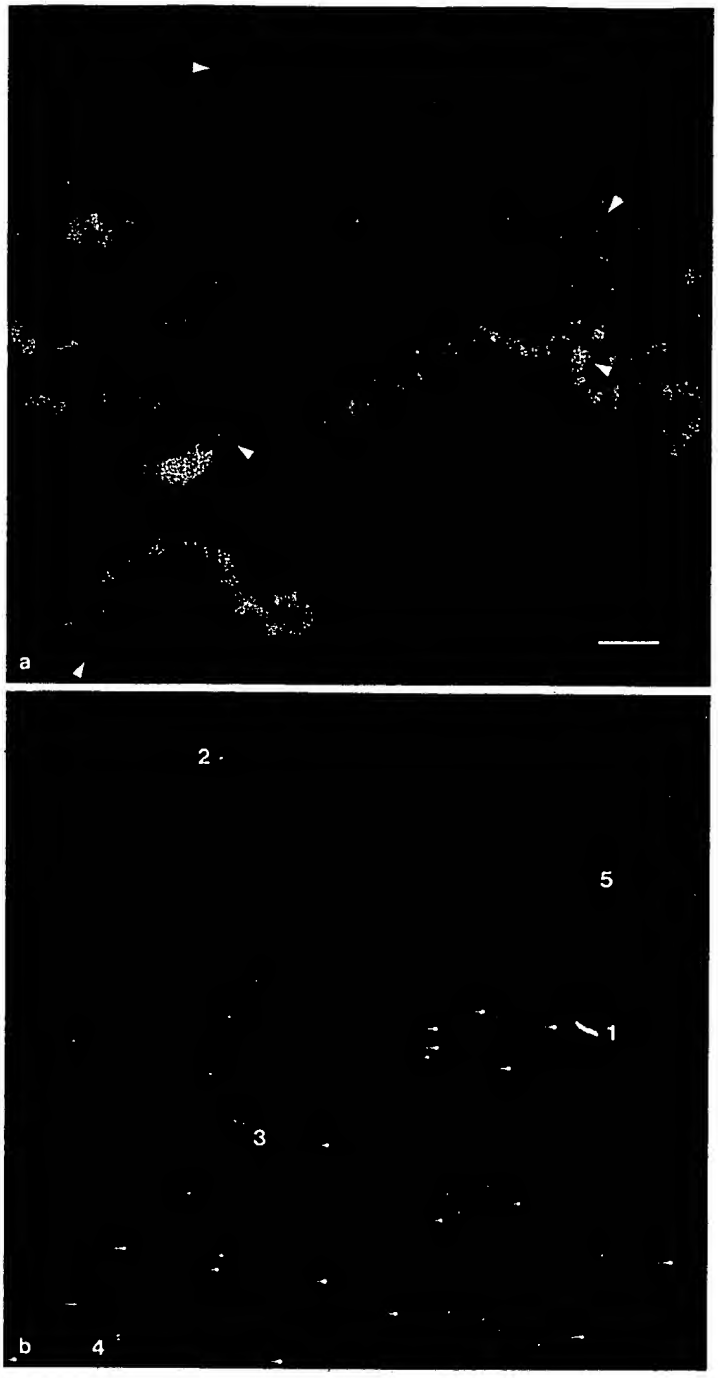

Fig. 3a and b. Hybridisation of rhodamine-labeled tRNA (Arg-2) to *D. melanogaster* chromosomes to an R_0t value of 26. Blue DAPI fluorescence (**a**) and red rhodamine fluorescence (**b**) are from the same field. Indicated in **b** are: *1* 56F; *2* 84F; *3* 42A; *4* 62A; *5* 52F

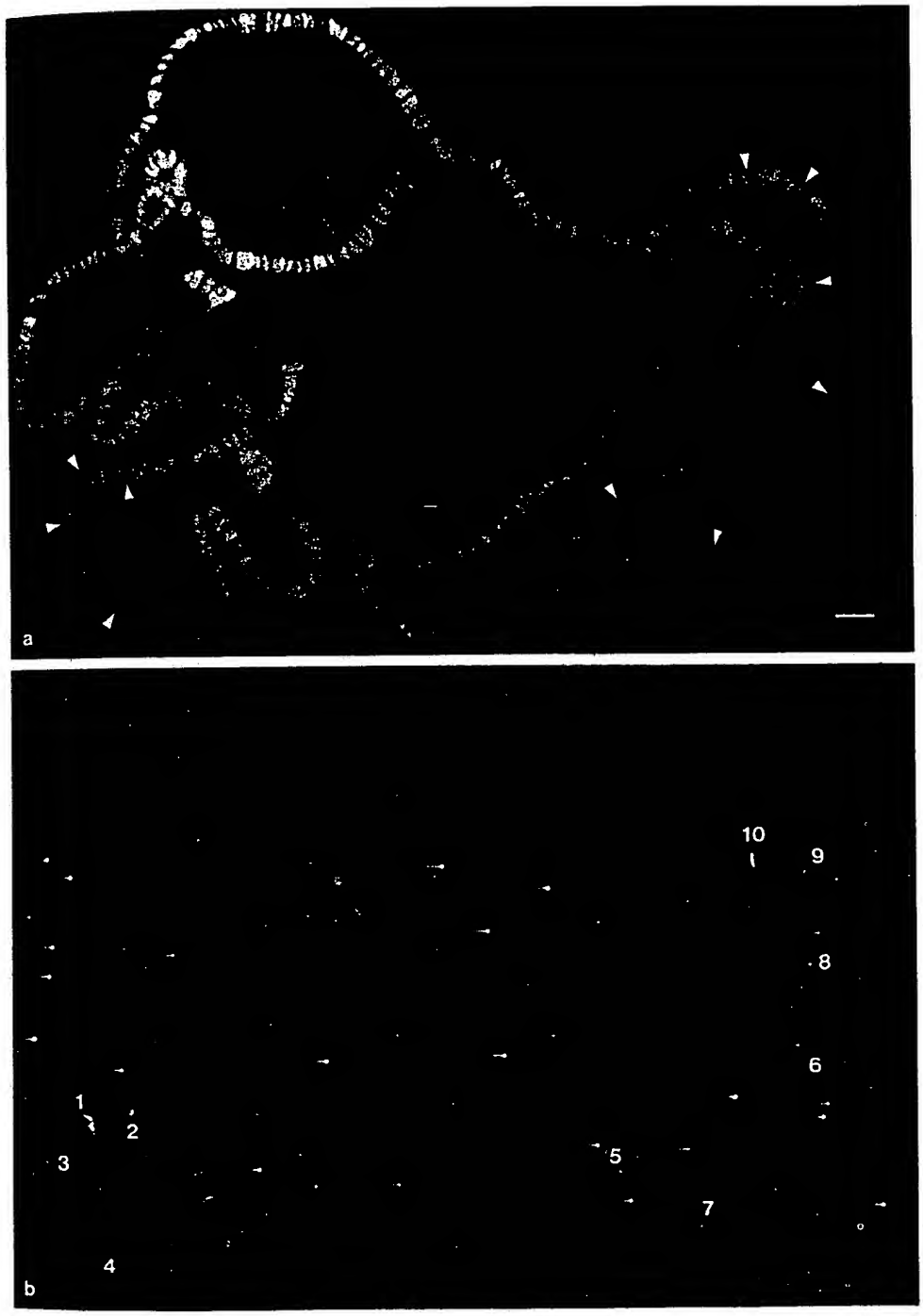

Fig. 4a and b. Hybridisation of rhodamine-labeled tRNA (Gly-3) to *D. melanogaster* chromosomes to an R_0t value of 20. Blue DAPI fluorescence (**a**) and red rhodamine fluorescence (**b**) are from the same field. Indicated in the figure are: *1* 56EF; *2* 57C; *3* 55E; *4* 53E; *5* 35BC; *6* 28D; *7* 22BC; *8* 92AB; *9* 96A; *10* 99A

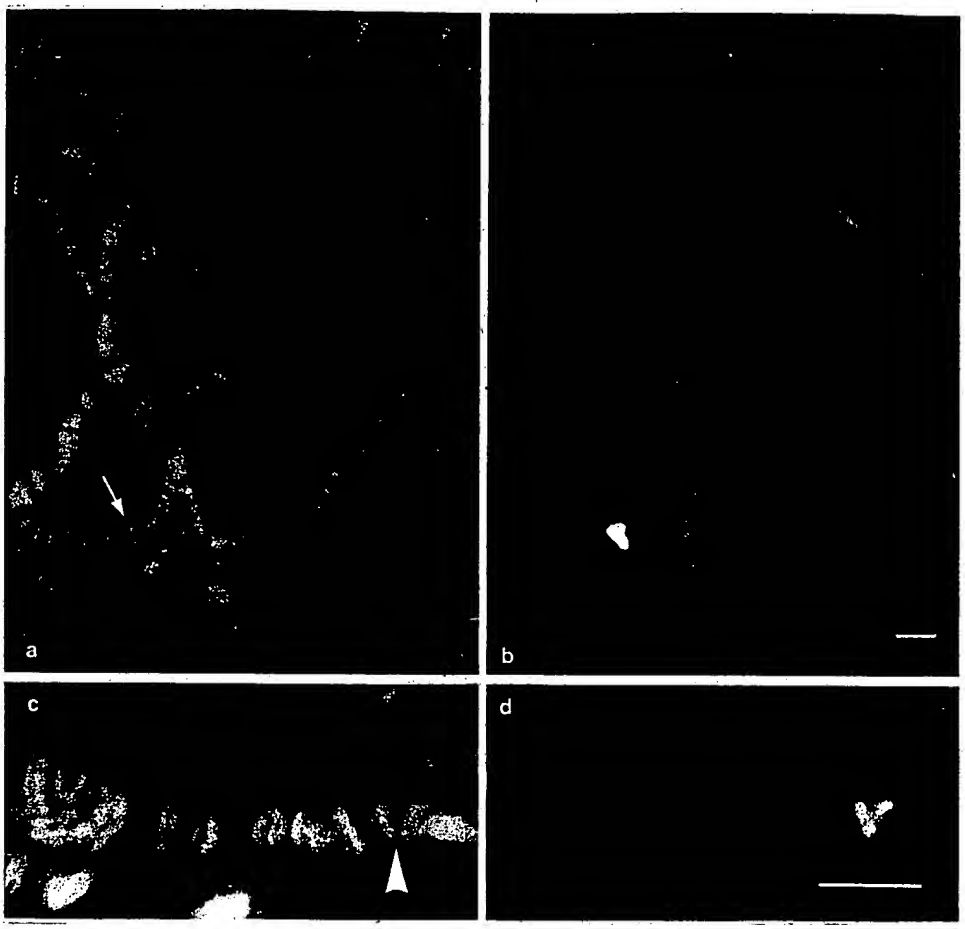

Fig. 5a–d. Hybridisation of rhodamine-labeled RNA complementary to clone cDm500 DNA containing histon genes of *D. melanogaster* to an R_0t value of 3.4. Blue DAPI fluorescence (**a, c**) and red rhodamine fluorescence (**b, d**)

site (Fig. 5) wereas no other region of the chromosomes nor the nucleolus were even faintly labeled. Figure 5b is overexposed on purpose, to show the absence of labeling at any site except 39DE. The labeled region was often found to be irregular in shape as is typical for this chromosome region (Fig. 5d) (see also Pardue et al., 1977).

Clone 232.1 cRNA

Rhodamine-labeled cRNA of the total plasmid 232.1 failed to label any site after hybridisation with *D. hydei* preparations to R_0t values of about 3. A faint fluorescent band was visible after R_0t values of 34 (not shown).

The rhodamine-labeled cRNA of the insert of plasmid 232.1 gave a positive label at 2–32A after hybridisation to $R_0t = 17–22$ (Fig. 6) and even a very faint fluorescence was often visible at locus 2–36A. With ^3H-labeled cRNA of plasmid

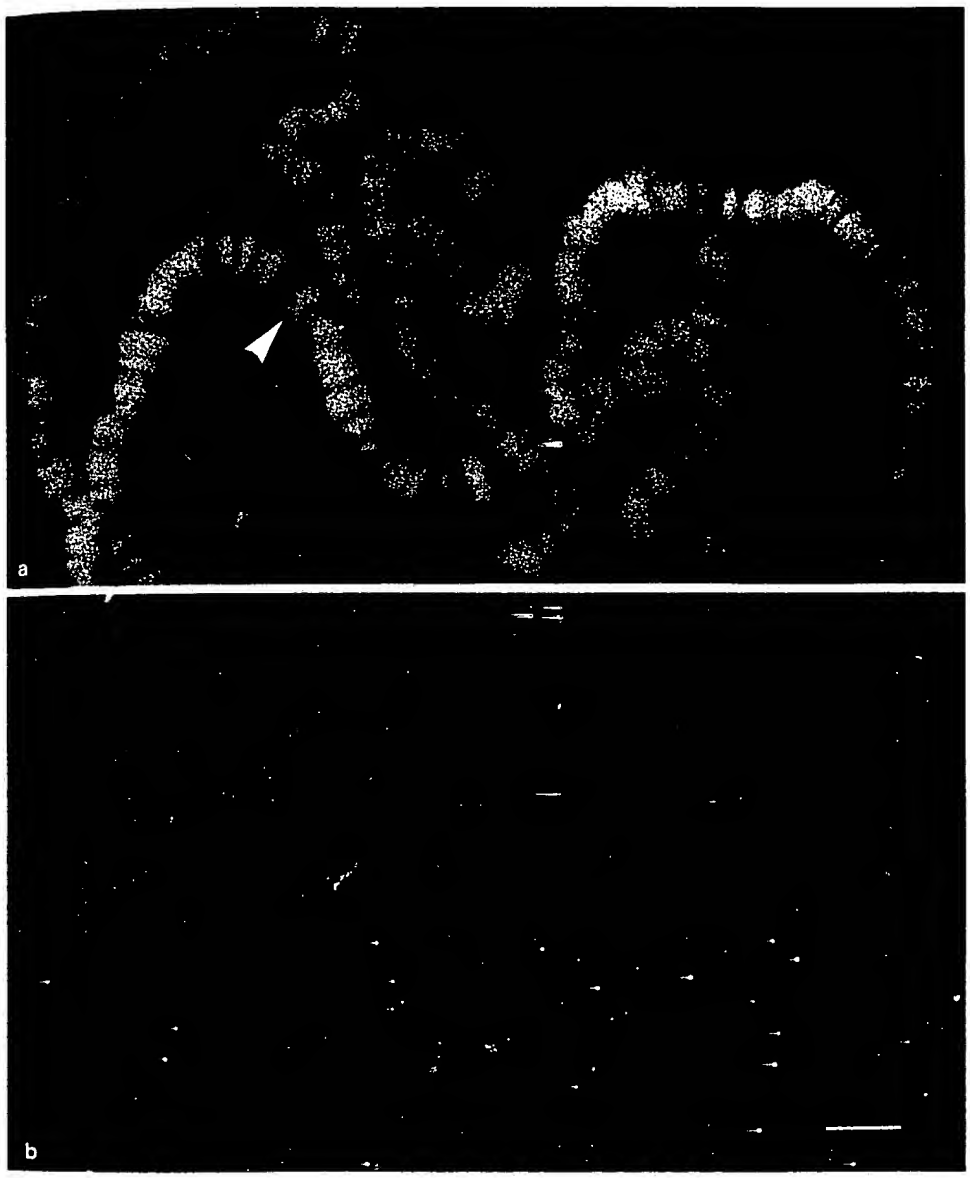

Fig. 6a and b. Hybridisation of rhodamine-labeled RNA complementary to the insert DNA of clone 232.1 containing 1 kbp of a *D. melanogaster* heat shock gene to *D.* hydei polytene chromosomes. Blue DAPI fluorescence (a) and red rhodamine fluorescence (b)

232.1 about 7 times fewer grains develop at this locus as compared to 2–32A (Peters et al., 1980). At $R_0t=368$ the non-specific background fluorescence was so bright that specific labeling was no longer visible.

The labeled site at 2–32A in many cases was not one sharp fluorescent band but consisted often of a pair of bands, one of which was always clear and sharp while the other, located at the proximal side of the 32A locus near the puff at 31C, was often fainter and appeared to be swollen (Fig. 6).

Clone 244.1 cRNA

Rhodamine-labeled cRNA of the total plasmid 244.1 failed to label any site after hybridisation with *D. hydei* polytene chromosome preparations to R_0t values of 5–7. Possible explanations for this are mentioned in the Discussion. At a R_0t value of 78 the background fluorescence was high, which might have "obscured" a weak fluorescent band. Peters et al. (1980) described that ^{3}H-labeled cRNA of this plasmid gave a clear labeling of locus 4–81B after in situ hybridisation and autoradiography for 60 days.

Non-specific Fluorescence

All preparations incubated with fluorochrome-labeled RNA showed variable fluorescence in cytoplasmic components. This did not interfere with the detection of specific hybridisation on chromosomes, provided the nuclei were well spread. The intensity of the cytoplasmic fluorescence depended on the concentration and batch of fluorochrome-labeled RNA used and it could not be blocked by unlabeled RNA. The cause lies probably in hydrophobic interactions of rhodamine with some cytoplasmic components. This is indicated by the extremely strong fluorescence of fat-body cells which were occasionally present on the squash preparations.

A dull uniform fluorescence without banding was nearly always present on polytene chromosomes, the intensity of which was proportional to concentration and batch of fluorochrome-labeled RNA used. In most cases this did not interfere with the detection of specific hybridisation.

Discussion

It is clear from the results presented in this paper that fluorochrome-labeled RNA can be used in cytochemical hybridisation to map the position of its complementary DNA in polytene chromosomes with high precision due to the high resolving power of fluorescence microscopy. Furthermore, the results are available faster than with cytochemical hybridisation using radioactively-labeled nucleic acids, because no exposure-time is needed for autoradiography.

Specificity

The specificity of the cytochemical hybridisation with fluorochrome-labeled RNA is best demonstrated by the labeling of only one locus, when RNA complementary to cloned histone genes (Fig. 5) or a single cloned heat shock gene (Fig. 6) is used. Using purified tRNA species, several sites are fluorescent in a pattern which coincides with the tRNA loci previously assigned by radioactive cytochemical hybridisation.

The occurrence of label at a few distinct other sites in the case of the purified tRNAs was probably caused by the relative high R_0t values used in these experiments. If we assume that our hybridisation rates are comparable to those of Szabo et al. (1977) (50% formamide/2 × SSC, 40° C) (which might be concluded from the data of Hutton (1977) and Casey and Davidson (1977) on the effect of formamide and salt concentration on the kinetics of duplex formation; but see

below) the $R_0t_{1/2}$ of a pure tRNA species is about 0.6 times the $R_0t_{1/2}$ of 5S rRNA (Szabo et al., 1977), or $0.6 \times 0.0022 = 0.0013$ mole sec/l.

There are two possible causes for the occurrence of label at additional sites: hybridisation of impurities or crosshybridisation to other tRNA genes. A contamination with a different tRNA species as low as 0.5% would at $R_0t = 26$ still hybridise for at least $100 \times R_0t_{1/2}$ thereby saturating its locus. On the other hand, Hoveman et al. (1980) demonstrated that the high degree of homology between *D. melanogaster* tRNAs was probably the explanation for the observed hybridisation of tRNA(Asn) to a DNA fragment containing only the tRNA(Ile) region, as determined by base sequence analysis. From the base sequences of tRNA(Glu-4) (Altweg and Kubli, 1980) and tRNA(Arg-2) (Silverman et al., 1979) it can be calculated with the rules of Hoveman et al. (1980) that in a region of about 31 bases (position 39–70 in both tRNAs) 77% homology exists. This might be enough sequence homology to give stable hybrids under the conditions we used, and might explain the observed labeling of both the Arg-2 and Glu-4 sites with rhodamine-labeled tRNA(Arg-2). Which of the two mechanisms, impurity or crosshybridisation, is the real cause of the observed additional bands with tRNA(Arg-2) might be investigated by competition hybridisation with purified tRNA(Glu-4).

Hybridisation Kinetics

If we assume that our hybridisation rates are comparable to those of Szabo et al. (1977), as stated above, than the R_0t values we used in our experiments were more than enough to saturate the DNA in the respective loci. In a few instances however, no fluorescence was observed, although one would expect the genes to be saturated (244.1 cRNA at $R_0t = 6$, 232.1 cRNA at $R_0t = 3$). This suggests that the hybridisation rate might be much lower at 23° C, 70% formamide/3 × SSC than at 40° C, 50% formamide/2 × SSC [the conditions used by us and Szabo et al. (1977), respectively]. The relative hybridisation rate may be estimated from the R_0t value needed to give a visible fluorescence at a locus were only few gene copies per haploid genome are present, for example a specific tRNA gene or a heat shock gene. It can be calculated (see below) that for these genes near saturation must be achieved before a local fluorochrome concentration is produced which is high enough to be visible. Visible fluorescence at these loci was only observed after about $100 \times R_0t_{1/2}$ or more. In theory about $10 \times R_0t_{1/2}$ is sufficient to saturate any locus (see Szabo et al., 1977). We conclude that the estimated hybridisation rate at our conditions is about 10 times lower than at the conditions used by Szabo et al. A further systematic investigation of this subject is needed, because at higher temperatures and lower formamide concentrations (conditions at which the hybridisation rate is higher) the fluorochrome-RNA bond becomes unstable which produces a decreased signal and increased background fluorescence.

Topological Resolution

The localising power of fluorescence microscopy as compared to autoradiography is illustrated well by the purified tRNAs, which produce very sharp fluorescent bands (Figs 3 and 4); the bands become only diffuse when the labeled region is somewhat puffed. Another example of the high resolving power is the

demonstration of a double band at locus 2–32A labeled with 232.1 insert cRNA (Fig. 6). With ^{3}H-labeled 232.1 cRNA and autoradiography it was never possible to unambigiously demonstrate this double band (Sondermeijer and Lubsen, unpublished observations).

Sensitivity

We determined in our fluorescence microscope using an agarose-bead model-system that 1000 rhodamine molecules per μm^2 are still visible against a black background (Bauman et al., 1981a). Renkawitz-Pohl (1978) determined that in *D. hydei* polytene chromosomes about 250 5S rRNA gene copies per haploid genome are present resulting in 250,000 gene copies per band. This is about the same value as for *D. melanogaster* (Szabo et al., 1977). Assuming that the in situ hybridisation efficiency under our conditions is also 10%–15% (Szabo et al., 1977, Cote et al., 1980) and given that the degree of labeling of the 5S preparation used is one rhodamine molecule per 40 nucleotides, we should obtain at least 78,000 fluorochrome molecules per band. The size of the band is about $1 \times 5\,\mu m$ which results in about 16,000 rhodamine molecules per μm^2. This is well above the limit of visibility of about 1000 rhodamine molecules per μm. This calculation can also be performed for a tRNA gene site. Dudler et al. (1980) determined that locus 84F contains three tRNA(Arg-2) gene copies per haploid genome. From this it can be calculated that 415 fluorochrome molecules can be expected at 84F (the number of tRNA gene copies (3) $\times$ the in situ hybridisation efficiency (0.15) $\times$ the degree of labeling (0.9) $\times$ the polyteny (1024) is $3 \times 0.15 \times 0.9 \times 1024$). This is in reasonable agreement with the lower limit of visibility of 1000 molecules per μm^2 considering the uncertainties in the calculations of, e.g., hybridisation efficiency.

It is of interest to compare the sensitivity of the fluorescence-microscopical hybridisation method to the autoradiographical method.

When the tRNA had been ^{3}H-labeled with a specific activity of 10^7 dpm/μg one silvergrain should have developed after an exposure time of 36 days. The efficiency of ^{3}H-autoradiography is taken to be 10%.

About one silver grain per day should have appeared with ^{125}I-labeling at 2×10^8 dpm/μg (e.g., Kubli and Schmidt, 1978) (autoradiographic efficiency 23%, Szabo et al., 1977). The 32 ± 13 grains found at 84F by Hayashi et al. (1980) with tRNA(Arg) at 1.5×10^8 dpm/μg after an exposure time of 34 days is in excellent agreement with this calculations.

Explicitly stated, the sensitivity of the present method is such that less than three tRNA gene copies are detectable in normal polytene chromosomes, which is 220,000 bp or less provided these are located in a single band or spot.

With clone 244.1 cRNA at R_0t values of about 6 no labeling was found at 4–81B, although approximatly 800,000 complementary bases should have been present (0.8 kbp insert $\times$ 1024 for polyteny). A visible fluorescence could only have been expected at this site when the hybridisation efficiency had been as high as 15%. But the R_0t value used was probably to low considering that only about 15% of the RNA was complementary to *Drosophila* DNA. Also the sequence divergence between *D. melanogaster* and *D. hydei* heat shock DNA at this locus might have caused a decrease in the hybridisation rate. Finally, the quality of fluorochrome

labeling of this batch of cRNA was probably low. These factors and the ones mentioned below determined whether visible fluorescence at a certain site might be expected.

Methodological Aspects

It is clear that a given gene is easier to detect using the fluorescence microscope when its DNA is in a condensed state resulting in a high local fluorescence of the hybrid. For autoradiographical detection on the other hand it is of advantage that the site is puffed which results in a spreading of silvergrains over a large area (compare localisation of tRNA (Arg-2) at 42A (Results section and Hayashi et al., 1980).

The sensitivity of the fluorescence microscopical method also depends on the degree of background fluorescence and/or nonspecific binding of fluorochrome molecules. The non-specific binding of fluorochrome molecules can be prevented by taking good care to purify the fluorochrome-labeled RNA. To remove rhodamine phenol extraction is very efficient. For other fluorochrome molecules, e.g. fluorescein, repeated ethanol precipitations are probably most effective. Hybridisation at higher temperatures than 25–30° C for prolonged periods (several days) is not recommended, because fluorochrome molecules that detach from the RNA will bind to the proteins in the preparation and cause a high background. At the same time the specific fluorescence is lowered.

The occurrence of labeling of bands by impurities in the RNA preparations can be prevented by performing the hybridisation at appropriate R_0t values (see Kubli and Schmidt, 1978) (thereby taking the possible lower hybridisation rate into account) or by the addition of non-fluorochrome labeled RNA. This competition by unlabeled RNA cannot be performed with the method of Rudkin and Stollar (1977) in which antibodies specific for RNA · DNA hybrids and immunofluorescence are used. It would only increase the unwanted labeling. This is a major drawback of that elegant and potentially sensitive method.

For a more detailed discussion of the factors governing the sensitivity of cytochemical hybridisation and the number of fluorochrome molecules expected after cytochemical hybridisation with rhodamine-labeled RNA we refer to Bauman et al. (1981 b). In general, the main factors concerning the number of fluorochrome molecules to be expected are:

1) the amount of DNA at the site after the hybridisation, 2) the bybridisation efficiency, 3) the degree of labeling of the RNA used (which is in general 0.7–0.9) and 4) the size of the RNA hybridised. Concerning the last point it can be remarked that because each RNA molecule is labeled with only one fluorochrome molecule it is necessary to degrade large RNAs to an average length of for example 50 nucleotides. The 3′-terminal phosphate groups that might be present, must be removed by phosphatase treatment before the labeling procedure.

The fluorochrome-labeling procedure itself requires several micrograms of RNA in order to minimize los of RNA. It is possible to add carrier RNA after the initial periodate oxidation and chromatography only when RNA is radioactively labeled and no UV absorbance measurements are needed to quantitate the degree of labeling.

One disadvantage of the described fluorochrome-labeled RNA method is that it

cannot be used in induced systems (in which in vivo puls-labeling is used) unless the newly synthesized RNA can be purified.

It is possible to measure by fluorometry the local fluorescence intensities of labeled bands in order to quantitate the amount of RNA hybridised. In the present study we did not measure local fluorescence values of polytene chromosomes, although we showed in another system that it is feasible (Bauman et al., 1981 b). The fluorescence intensities can be measured directly in the fluorescence microscope or indirectly by the scanning-microdensitometric method of Van der Ploeg et al. (1977). In this method photographic negatives are used. Calibration in absolute amounts of RNA hybridised can be accomplished by the use of a highly purified RNA for example 5S rRNA, which is both radioactive- and fluorochrome-labeled and performing both fluorescence measurements and autoradiography of the same band.

The sensitivity of the fluorescence microscopical hybridisation procedure described in this paper can be increased, for example by an immunocytochemical amplification method described by Schmitz and Kampa (1979). The application of this method for in situ hybridisation has been investigated and will be described elsewere (Bauman, Wiegant, van Duijn, in preparation).

It can be concluded that the use of fluorochrome-labeled RNA for the detection of specific RNA species by cytochemical hybridisation has the advantage of high speed, high resolving power, the possibility of quantifying by fluorometry, the relative easiness with which the labeling is performed and the use of commercially available and non-radioactive materials. The sensitivity is in general better than ^{3}H-autoradiography when only in-vivo labeled RNA is available or equal to or less when highly radioactively labeled RNA can be prepared in vitro. However, the use of immunofluorescence amplification methods still might improve the sensitivity of the method.

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